

From the Department of Zoophysiology, the Department of Pharmacology and the
Department of Obstetrics and Gynecology, University of Lund, Sweden.

**CALCIUM BINDING BY SARCOPLASMIC RETICULUM AND MITOCHONDRIA
ISOLATED FROM SKELETAL AND SMOOTH MUSCLES AND ITS RELATIONSHIP
TO CONTRACTION**

By

Satish Batra

Lund 1973

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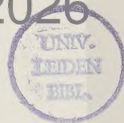
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This thesis is based on the following reports, which will be referred to by their Roman numerals in the text.

- I. Batra, S. (1972). The effect of sodium and potassium on calcium uptake by frog skeletal muscle mitochondria and vesicles. *Can. J. Physiol. Pharmacol.* 50, 1157-1161.
- II. Batra, S. (1973). The effects of zinc and lanthanum on calcium uptake by mitochondria and fragmented sarcoplasmic reticulum of frog skeletal muscle. *J. Cell. Physiol.* (in press).
- III. Batra, S. (1973). The effects of drugs on calcium uptake and calcium release by mitochondria and sarcoplasmic reticulum of frog skeletal muscle. *Biochem. Pharmacol.* (in press).
- IV. Batra, S. (1972). The role of calcium binding by subcellular particles in the contraction and relaxation of the myometrium. *Am. J. Obstet. Gynec.* 112, 851-856.
- V. Batra, S. and Timby, L. (1971). ATP requirement in the course of calcium uptake by human myometrial mitochondria. *FEBS Letters* 18, 238-240.
- VI. Batra, S. and Bengtsson, L.Ph. (1972). Inhibition by diethyl stilbestrol of calcium uptake by human myometrial mitochondria. *Europ. J. Pharmacol.* 18, 281-283.
- VII. Batra, S. (1973). The effect of some estrogens and progesterone on calcium uptake and calcium release by myometrial mitochondria. *Biochem. Pharmacol.* 22, 803-809.
- VIII. Batra, S. (1973). The role of mitochondrial calcium uptake in contraction and relaxation of the human myometrium. *Biochem. Biophys. Acta*, 305, 428-432.

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INTRODUCTION

Skeletal muscle

The importance of Ca ions in muscle physiology was recognized as early as 1883 when Ringer showed that frog ventricles will fail to contract in Ca free saline and that spontaneous contraction will resume upon the addition of Ca. The importance of intracellular Ca for contraction became apparent from the experiments of Heilbrunn and Wiercinski (1947) who injected various ions into single muscle fibres; Ca was the only physiologically acting cation which would cause shortening of the muscle fibre.

By 1952, considerable data on Ca involvement in muscle behaviour had accumulated and Sandow (1952) postulated that the membrane depolarization during the action potential liberated some substance, probably Ca from the membrane structure itself into the myoplasm. This Ca would then move into the interior of the fibre by a mechanism other than diffusion, possibly an exchange reaction in order to activate the entire fibre. Sandow hoped, by suggesting this exchange-diffusion mechanism to obviate the difficulty emphasized by A.V. Hill (1948) i.e., simple diffusion of any activating substance is too slow to account for the extremely rapid development of full activity observed in a twitch. At this time Sandow also described his ideas on excitation-contraction (E-C) coupling.

E-C coupling was defined by Sandow (1965) as a function of the muscle fibre in which an electrical depolarization of the plasma membrane initiates a sequence of reactions that causes mechanical activation of the contractile myofibrils lying within the membrane. To complete the cycle, however, a process of relaxation must follow. By far, the greatest amount of work on the elucidation of these processes has been done on fast skeletal muscle fibres, and it is now well established that Ca plays a key role in the regulation of this function (e.g. Ebashi and Endo, 1968; Sandow, 1970).

It is generally accepted that in fast skeletal muscle, an effective stimulus

releases activator Ca from the internal stores of sarcoplasmic reticulum which directly activates contraction in the myofibrils. Ca is then reaccumulated by the sarcoplasmic reticulum by an energy dependent process (Sandow, 1965; Weber, 1966; Ebashi and Endo, 1968). To support this hypothesis, experimental evidence has been presented by many workers showing that isolated fragments of the sarcoplasmic reticulum from skeletal muscle are able to accumulate Ca efficiently and rapidly from the surrounding medium (e.g. Hasselbach, 1964; Weber, 1966). Mitochondria from muscle and other tissues have also been shown to be capable of binding considerable amounts of Ca in vitro (Lehninger, Carafoli and Rossi, 1967; Dransfeld, Greeff, Schorn and Ting, 1969; Carafoli, Tiozzo, Rossi and Lugi, 1972).

Although depolarization of the fibre membrane is most probably the initial physiological step for inducing a muscle contraction, a number of agents under certain experimental conditions are capable of eliciting contraction in a completely depolarized muscle (Axelsson and Thesleff, 1958; Isaacson and Sandow, 1967; Andersson, 1972). Studies on such preparations have not only helped in elucidating the mechanism of drug action on the contractile responses of muscle but have also provided additional support for the view that the rise in myoplasmic Ca which precedes contraction is a consequence of its release from the intracellular stores of muscle.

Investigations on the function of the sarcoplasmic reticulum have been pursued largely through biochemical studies of rabbit or rat muscle whereas studies on the electromechanical aspects of muscle contraction have mainly been performed on frog skeletal muscle. This has been generally the case for investigations on actions of potentiators of muscular contraction such as zinc, cadmium, caffeine and quinidine (see Sandow, 1965, 1970). Furthermore, the experimental conditions used in the biochemical studies have varied considerably. Thus, it is not surprising to find controversies in the physiological interpretation of results obtained from isolated fractions. It is clear that both types of studies should be carried out on muscle from the same species in order to correlate

physiological and biochemical observations. Furthermore, in view of the fact mentioned above that mitochondria from muscle, in addition to sarcoplasmic reticulum, can take up large quantities of Ca, the question of their relative contribution to the control of myoplasmic Ca should also be examined. These were then the main objects of the present investigations on the skeletal muscle subcellular fractions.

Smooth muscle

It is generally agreed that less is known about the mechanism of E-C coupling in mammalian smooth muscles (including the myometrium) than about skeletal muscle. There are several reasons for this which are most appropriately summed up by Kao who states:

"Many factors are responsible for this difference. Some of them are due to the vagaries of smooth muscle preparations, which discourage even the best intentioned physiologists, and others are due to additional prejudices of physiologists, who avoid working with smooth muscles. Understandably, it is very difficult to analyze the factors in the latter category, but many of them can certainly be traced to factors in the former. The so-called vagaries of smooth muscle are undoubtedly just reflections of our ignorance of them. For too long we have approached various smooth muscles with the expectation that they will behave, respond and possess the same properties as skeletal or cardiac muscle."

There is at the present time a general agreement on the universal role of Ca in smooth muscle contraction (e.g. Daniel, 1965; Somlyo and Somlyo, 1968; Lullman, 1970) in spite of the relatively slow progress made in our understanding of the mechanism regulating tension development in smooth muscles. In recent years a vast amount of literature has accumulated on the subject and consider-

able evidence indicates that Ca is importantly involved in all three stages of smooth muscle contraction, namely: 1) excitation or electrical events, 2) contraction or mechanical events and 3) the link between electrical and mechanical events (i.e. E-C coupling).

The existence of intracellular Ca translocation in uterine smooth muscle comparable to the sarcoplasmic reticular system of skeletal muscle has been postulated from studies on the distribution and movements of Ca in the rat myometrium (Van Breemen, Daniel and Van Breemen, 1966). On the other hand a diffusion of extracellular Ca into cells and intracellular Ca into the extracellular space have been suggested as alternative mechanisms of contraction and relaxation. These suggestions were made primarily because of the small amount of endoplasmic reticulum, the short diffusion distance in smooth muscle and the slow rates of contraction and relaxation in these tissues. However, several types of smooth muscle bathed in Ca free media contract in response to drugs for some time after the loss of the contractile response to high potassium or electrical depolarization. Examples of this phenomenon are the effects of norepinephrine on rat tail artery (Hinke, 1965) and of acetylcholine on rat uterus (Edman and Schild, 1962), taenia coli (Durbin and Jenkinson, 1961) and toad stomach (Sparrow and Simmonds, 1965). A related property of a number of smooth muscles is their greater maximal contractile response to drugs than to complete depolarization with potassium. The maximal responses of vascular smooth muscle to norepinephrine (Waugh, 1962; Hiraoka, Yamagishi and Sano, 1968; Somlyo and Somlyo, 1968) and the response of uterine (Edman and Schild, 1962) intestinal (Evans, Schild and Thesleff, 1958) and trachial (Somlyo and Somlyo, 1970) smooth muscle to acetylcholine are good examples. These characteristics of smooth muscle could be explained by the ability of the drugs to translocate Ca into the cytoplasm from a compartment inaccessible to membrane depolarization. Thus the importance of studies dealing with Ca binding and release by the subcellular particles of smooth muscle is emphasized.

Recently an ATP dependent Ca binding by mitochondria and microsomes from cow uterine smooth muscle and from rat myometrium has been demonstrated by Carsten (1969) and Batra and Daniel (1971), respectively. Several qualitative and quantitative differences were noted between the results of the two studies. In addition, structural differences could be seen between electron micrographs prepared from microsomal fractions of rat myometrium and cow myometrium indicating an important species difference in the characteristics of this muscle. In view of this and the fact that human myometrium forms the bulk of uterine tissue, easily separable from the rest of the uterus, the present study examined the role of mitochondrial and microsomal Ca uptake in contraction and relaxation of the human myometrium.

METHODS

A. Skeletal Muscle

1. Preparations of subcellular particles

Approximately 5 gram of muscle from frogs (*Rana temporaria*) was homogenized in ice-cold KCl (150 mM) and imidazole (5 mM) solution (pH 6.8) with an Ultraturrax homogenizer at 20000 rpm 5 times for 3 seconds periods with intermittent pauses of 30 sec. The homogenate was centrifuged at 900 x g for 10 min and the pellet discarded. The supernatant was filtered through two layers of gauze and centrifuged at 4000 x g for 15 min to remove mitochondria. The supernatant was then centrifuged at 8500 x g for 15 min and the pellet discarded since electron microscopic examination revealed a marked cross contamination in this material. The remaining supernatant was spun at 34000 x g for 45 min to obtain fragmented sarcoplasmic reticulum (FSR). The pellets of mitochondria and FSR thus obtained were suspended in 0.6 M KCl and respun. The sediment from each was then washed with the same medium as used for homogenization and suspended in it to a protein concentration of 1 to 1.5 mg per ml. Preliminary experiments showed that use of the above scheme of centrifugation speeds and durations to separate the subcellular particles gave mitochondria and FSR with low contamination and high degree of reproducibility. Approximately 1 mg of FSR or mitochondrial protein per gram muscle was obtained. The above procedure was carried out at 2-4°C. The method using deionized water as a medium for homogenization and isolation (Ogawa, 1970) had to be abandoned in view of the structural changes in mitochondria and the lack of purity of the isolated fractions (I).

2. Calcium uptake measurements

The principle of the method is simple. Briefly, the particulate fraction (FSR or mitochondria), is incubated under the given conditions in an appropriate medium containing Ca with the tracer Ca^{45} . At a predetermined time the particu-

late fraction is separated from the medium. There are two methods available for this separation: (a) centrifugation and (b) filtration through filter papers with pore size small enough to retain the particulate fraction. The second method besides being simpler has the obvious advantage namely, a virtually instantaneous termination of the reaction. This method (filtration method) using Millipore filters was employed in the present study.

Generally the assay medium consisted of 150 mM KCl, 15 mM imidazole buffer (pH 7.0), 5 mM MgCl_2 , 1 mM ATP and 0.1 or 0.01 mM Ca with $2.5 \mu\text{C } ^{45}\text{CaCl}_2$. The reaction was started by the addition of 0.3 to 0.4 mg of mitochondrial or FSR protein in a total volume of 1 ml. The reaction was carried out either at 20°C or 4°C . At specified time intervals, usually 0.4 ml of the reaction mixture was filtered through Millipore filters (0.45μ diam.). Blanks which lacked only the particulate material were filtered simultaneously. Ca uptake was determined by subtracting the amount of radioactivity in the filtrate of the sample from that of the blank. Radioactivity was determined by a Packard Tri-Carb liquid scintillation counter. Correction for quenching was made by the channel ratio method.

3. Calcium release measurements

The fractions were allowed to take up Ca for 5 minutes in a medium described under Ca uptake experiments, and 0.1 ml of the appropriate concentration of the drug to be tested was added. After 2 and 5 minutes aliquots of the reaction mixture were filtered through the Millipore filters. Ca release was calculated from the increment in the radioactivity of the filtrate after the addition of the drug. Appropriate controls without the drug were run simultaneously.

4. Enzyme assays

Succinic dehydrogenase activity was determined spectrophotometrically with phenazine methosulphate-2,6-dichlorophenolindophenol (PMS-DCIP) as the electron acceptor system described by Arrigoni and Singer (1962). The reaction mixture contained potassium phosphate buffer (pH 7.6), 1 mM KCN (freshly neutralized), 0.75 mM CaCl_2 and 0.5 mg of mitochondrial or FSR protein. The reaction was started by successive addition of (final concentration) 20 mM succinate, 50 μM DCIP and 1 mg/3 ml of PMS and the decrease in absorbance at 600 nm was measured with a Hitachi spectrophotometer.

ATPase activity was assayed by measuring the Pi content of the filtrates according to the method of LeCocq and Inesi (1966). Protein concentration was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a standard.

5. Electron microscopy

Preparations were fixed in a solution containing 3 % glutaraldehyde, 0.4 mM CaCl_2 and 100 mM phosphate buffer (pH 7.0). Sections were cut on an LKB ultratome after dehydration in ethanol and embedding in Epon. After staining with lead citrate and uranyl acetate they were examined in a Philips electron microscope (EMU 300).

B. Smooth muscle

1. Isolation of mitochondrial and microsomal fractions

Pieces of uterine tissue were removed at operations, usually Caesarean-sections, and collected in Krebs-Ringer bicarbonate solution (Batra and Daniel, 1970). The endometrium was carefully removed and the myometrium was cut into strips 2-3 mm wide and homogenized in ice-cold sucrose (0.25 M) and histidine (5 mM) solution. A greater degree of homogenization than used for skeletal

muscle was needed due to the tough nature of uterine tissue. A five second period of homogenization per gram wet weight of tissue was found to be adequate at a speed of 20000 rpm (Ultraturrax homogenizer). Subcellular fractions from the homogenate were separated by differential centrifugation as described in paper IV and VI.

2. Calcium uptake and Calcium release

The basic technique for measurement of Ca uptake and Ca release was the same as that described for skeletal muscle experiments except that incubation time was longer due to the slower rate of Ca uptake in these fractions. Preliminary observations indicated that mitochondria isolated from this tissue were extremely unstable to ageing. In subsequent experiments, greater care was taken to reduce the time between isolation of the mitochondria and Ca uptake measurements. Other details and any variations from the above general description of the methods are given in separate reports (I-VIII).

RESULTS AND DISCUSSION

A. Skeletal muscle

1. Isolation of the subcellular fractions

Using the technique of Ogawa (1970) who recommended deionized water for the isolation of FSR, it was found that the Ca uptake values were not as high as claimed by this author. Further, there were structural alterations in mitochondria. Due to this difficulty a comparative study of Ca uptake by mitochondria and FSR was not feasible with the use of Ogawa's method. The results of these experiments, however, provided some explanation for the contradictory reports on the effect of sodium and potassium on Ca uptake by FSR and mitochondria (see I). In subsequent experiments KCl-imidazole solution was used for homogenization and isolation of the subcellular fractions (II and III). A scheme (described under methods) for their separation was developed after rigorous trials using varying centrifugation speeds and durations for the separation of the subcellular fractions.

2. Characterization of the subcellular fractions

The fractions as characterized by succinic dehydrogenase assay and by selective metabolic inhibitors for mitochondria, appeared to be relatively free from cross contamination (II). This was further supported by the electron microscopic examination which also revealed that the majority of mitochondria were intact.

3. Calcium uptake by FSR

The results showing that FSR has a high affinity for Ca and that Ca is bound at a considerable rate are in general agreement with the findings of others on rabbit FSR (Weber and Herz, 1968; Carvalho, 1968 a, b; Ogawa, 1970). A calculation based on an estimate of the total amount of FSR and the myo-

plasmic Ca level during activation of muscle showed that FSR would fulfil the requirement for a relaxing system in skeletal muscle. These observations are discussed comprehensively in relation to the regulation of myoplasmic Ca in vivo during the contraction-relaxation cycle. The participation of mitochondria in controlling the myoplasmic Ca was also considered (II).

4. Calcium uptake by mitochondria

The results demonstrated (II and III) that the Ca uptake capacity of mitochondria was as high as that of FSR. Since the mitochondrial yield was equal to that of FSR, the uptake capacity of mitochondria per gram muscle also was as high as that of FSR.

The kinetics of mitochondrial Ca uptake, however, differed markedly from FSR since it was considerably slower, and not sensitive to very low (below $4 \times 10^{-6}M$) concentrations of Ca in the medium. These results indicated therefore that mitochondrial Ca uptake would probably not serve as a mechanism for relaxation in skeletal muscle. However, as thoroughly discussed in paper II, the participation of mitochondria in controlling myoplasmic Ca could not be ruled out under certain conditions, e.g., during high intracellular Ca levels.

5. Effects of zinc and lanthanum

Since zinc concentrations ($5-10 \mu M$) which maximally potentiate twitch in skeletal muscle fibers (Edman, Grieve and Nilsson, 1966), failed to influence Ca uptake by either mitochondria or FSR, it was concluded that the potentiating effect of zinc must be mediated by an action other than that on mitochondria or sarcoplasmic reticulum. This is in agreement with the recent electrophysiological observations on single fibers (Edman, Grieve and Nilsson, 1966) indicating that the effect of zinc in potentiating muscle twitch must be mediated primarily through an action on the cell membrane (see also Taylor, Preiser and Sandow, 1972).

Lanthanum, recently introduced as a tool for blocking the movements of Ca across the cell membrane (Van Breemen, 1969; Van Breemen, 1970), was used by Weiss (1970) to elucidate the role of Ca in E-C coupling in frog skeletal muscle. This author showed that lanthanum (0.1 mM) in the external medium prolonged the relaxation time of potassium contracture considerably. This effect of lanthanum has been confirmed by Andersson and Edman (1973) on single muscle fibers. In fact these workers were able to show a prolongation of the potassium contracture by lanthanum at a much lower concentration (10 μ M) than reported by Weiss. The present results on isolated fractions showed that lanthanum in very low concentrations (5 μ M) greatly reduced both the rate and capacity of Ca uptake by mitochondria. Lanthanum in this concentration inhibited only the rate of Ca uptake by FSR when the medium Ca was low but inhibited both the rate and the capacity when the medium Ca was high. These observations were explained by postulating 1) interference of lanthanum with the transport of Ca to its binding sites and 2) the existence of two types of binding sites for cations in the isolated preparation of FSR, one specific and the other non-specific for Ca (see paper II for a detailed discussion). The question of whether the prolongation of the potassium contracture by lanthanum can be explained by an action on the intracellular Ca translocation could not be resolved. The main difficulty was posed by the uncertainty of the extent to which lanthanum could penetrate the fibre membrane. However, the prolongation of relaxation can be explained by the slower Ca uptake by FSR observed in the presence of lanthanum and by the assumption that a small proportion of lanthanum in the extracellular medium was able to enter the muscle fibre during a potassium contracture and was free in the myoplasm.

6. Effect of drugs

This is the first study showing the comparative effects of drugs such as quinidine, chlorpromazine and caffeine on Ca uptake by mitochondria and FSR. The effects of individual drugs on Ca uptake and Ca release by the two sub-cellular fractions will be discussed briefly in the following section.

Quinidine

Quinidine in concentrations of 1-2 mM which have been shown to cause contracture in the living muscle (Isaacson, Yamaji and Sandow, 1970; Andersson, 1972) released a considerable amount of Ca from mitochondria but had little effect on Ca release from FSR. The uptake of Ca both by mitochondria and FSR was inhibited by higher concentration (1 or 2 mM) of quinidine but the inhibition of mitochondrial Ca uptake was much greater. With lower concentrations (0.4 mM), there was no significant effect on Ca uptake by FSR while 48 % inhibition of mitochondrial Ca uptake was observed. These results suggest that quinidine contractures are probably induced by releasing Ca from mitochondria. This suggestion is interesting as well as provocative in view of the fact that little importance is attached to the role of mitochondrial Ca uptake in skeletal muscle contraction and the prevailing dogma for an absolute role of sarcoplasmic reticulum in controlling myoplasmic Ca. However, as discussed thoroughly in paper III, there is no reason to assume that agents which have been implicated in the release of Ca from an intracellular site would do so only from Ca stores in sarcoplasmic reticulum and not from the stores in mitochondria. After all, mitochondria in vitro have a considerable capacity to store Ca.

Chlorpromazine

Chlorpromazine inhibited Ca uptake by mitochondria and by FSR. However, the inhibition of mitochondrial Ca uptake was greater than that of FSR. High concentrations of chlorpromazine (0.1 mM) released Ca from both FSR and mito-

chondria. The results of the present in vitro study suggest that a high concentration of chlorpromazine reaching the myoplasm might release Ca both from mitochondria and sarcoplasmic reticulum whereas lower concentrations would only inhibit the accumulation of Ca. The most significant finding with this drug, as with quinidine was the concentration-dependent duality of action of these drugs. This is the first report which shows that Ca uptake can be inhibited by low concentrations of the drugs while Ca release is only induced after a critical concentration of the drug is reached. The concentrations of the drugs, which released Ca either from mitochondria or FSR or both, were strikingly close to those reported to induce contracture in the intact muscle (Axelsson and Thesleff, 1958; Isaacson and Sandow, 1967; Isaacson, Yamaji and Sandow, 1970; Andersson, 1972).

Caffeine

Caffeine caused a greater inhibition of Ca uptake by mitochondria than by FSR. However, in contrast to quinidine, caffeine released Ca from FSR and not from mitochondria. This suggests that the contracture producing effect of caffeine is probably due to a release of Ca from sarcoplasmic reticulum, which is in agreement with suggestions made in the literature (Weber and Herz, 1968; Ogawa, 1970; Sandow, 1970). Several authors have reported differences in the contractures produced by quinidine and caffeine (Benoit, Carpeni and Przybyslawski, 1964; Isaacson, Yamaji and Sandow, 1970; Huddart, 1971). To explain this, Huddart (1971, 1972) has recently speculated that the two drugs released Ca from different sites in the sarcoplasmic reticulum. The results of the present study provide evidence that Ca is, in fact, released from different intracellular stores, i.e., mitochondrial in the case of quinidine and sarcoplasmic reticular in the case of caffeine.

B. Studies on human myometrium

Mitochondria and microsomes isolated from human myometrium were capable of binding Ca in the presence of ATP. Before discussing the Ca uptake properties of these fractions, it is worthwhile to note that the microsomal fraction from the myometrium may be considered a counterpart of FSR from skeletal muscle because they can be isolated by similar centrifugal forces. The microsomal fraction of uterine smooth muscle has, in fact, been referred to as sarcoplasmic reticulum by Carsten (1969). In the opinion of the present author this might be premature since a classical sarcoplasmic reticulum as found in the skeletal muscle and which is implicit by its designation, has not yet been unequivocally demonstrated in uterine smooth muscle.

1. Calcium uptake by the microsomal fraction

Although considerable amount of Ca was taken up by this fraction, it was much smaller than that bound by mitochondria. This was true both for the rate and the total amount of Ca taken up. Ca uptake capacity expressed in terms of the wet weight of tissue was about 5 times lower than that of mitochondria (IV). The rate of ATP hydrolysis during Ca uptake by this fraction was also lower than that by mitochondria. Other significant observations on microsomal Ca uptake were as follows.

(a) Oxalate which enormously increases Ca uptake by skeletal and cardiac muscle microsomes (Hasselbach, 1964; Fanburg and Gergely, 1965; Weber, 1966), had no effect on Ca uptake by myometrial microsomes.

(b) In skeletal and cardiac muscle microsomes, a Ca stimulated ATPase (extra ATPase) has been shown (Hasselbach, 1964; Fanburg, Finkel and Martonosi, 1964). Also Carsten (1969) has reported Ca stimulated ATPase in cow myometrium microsomal fraction. In the present study, there was no detectable extra ATP splitting by Ca which is in agreement with a previous report on rat myometrium (Batra and Daniel, 1971). In this regard microsomal Ca uptake by

rat and human myometrium (but not cow myometrium) resembles Ca uptake by kidney (Palmer and Posey, 1970) and red skeletal muscle microsomes (Streter, 1964; Harigaya, Ogawa and Sugita, 1968).

2. Calcium uptake by mitochondria

Since mitochondria from liver and cardiac muscle are able to take up Ca in the presence of a respiratory substrate such as glutamate or succinate (Lehninger, Carafoli and Rossi, 1967; Dransfeld, Greeff, Schorn and Ting, 1969; Lee, Hong and Kang, 1970), it was of interest to study the respiration-dependent Ca uptake by myometrial mitochondria. It was somewhat surprising to find that in contrast to liver or cardiac mitochondria, mitochondria from human myometrium were unable to accumulate Ca in the absence of ATP even when succinate was present (V). ATP produced a considerable increase in uptake. Oligomycin, an inhibitor of the transfer of energy from ATP to mitochondrial metabolism, completely abolished ATP supported Ca accumulation by the myometrial mitochondria. However, Ca uptake in the presence of ATP and succinate was only partially inhibited. This led to the conclusion that ATP does not only support Ca uptake by these mitochondria but also plays an essential role in the Ca uptake supported by energy from respiration. Similar observations were recently reported by Tjioe, Bianchi and Haugaard (1970) who studied brain mitochondria.

3. Effect of estrogen and progesterone

Both estrogen and progesterone have long been known to affect the muscular contraction of the uterus (e.g. Kumar, 1967). The mechanism by which these hormones influence the contractile activity of the uterus is not understood. Recent studies on the binding of these hormones to uterine tissue have indicated that they penetrate the uterine cell, rapidly (Evans and Hahnel, 1971).

Estrogens in small concentrations inhibited Ca uptake by mitochondria. Diethyl stilbestrol (DES) was found to be the most potent inhibitor of Ca uptake followed by ethinyl estradiol and estradiol-17 β (VII). Ca uptake by microsomes was little affected by DES (VI). Progesterone caused only an insignificant ($p < 0.05$) inhibition of Ca uptake. The effect of DES on Ca uptake by cardiac mitochondria was also tested. It was found that these mitochondria were less sensitive to the inhibition by DES than myometrial mitochondria. DES was also found to release Ca stored by mitochondria (VII).

The mechanism by which estrogens lower Ca binding by mitochondria is probably not a consequence of the inhibition of mitochondrial ATPase since the hydrolysis of ATP in the presence of estrogens remained unchanged. Estrogens were able to release the stored Ca from mitochondria. Thus, it was suggested that estrogens influence Ca transport in mitochondria by causing the mitochondrial membrane to be "leaky" to the cations.

4. Role of mitochondrial Ca uptake in contraction and relaxation of the myometrium

The large Ca binding capacity of the mitochondria and relatively selective inhibition of Ca uptake by estrogens led to the suggestion that these organelles may play a significant role in controlling the intracellular Ca concentration and thereby affect contraction and relaxation of the muscle. However, in order to judge whether the mitochondrial Ca uptake system studied in vitro is able to regulate relaxation by controlling Ca activity in the myometrium, two important questions were examined:

1. Can mitochondria lower the external Ca concentration to less than 10^{-7} M?
2. Can the rate of Ca uptake by the mitochondria account for the speed of relaxation?

The results show (VIII) that in contrast to microsomes, mitochondria from human myometrium were able to lower the Ca concentration below 10^{-7} M in a

medium which had contained 10^{-5} M. These data may mean that microsomal Ca uptake does not contribute to the regulation of cytoplasmic Ca in this tissue but caution must be exercised in relating data from an isolated system to intact tissue. The results of this in vitro study, however, show that mitochondria from human myometrium not only have the ability to lower the Ca concentration in the medium below the threshold (10^{-7} M) needed for the activation of the contractile system (Weber, 1966; Ebashi and Endo, 1968) but can do so with a speed comparable to that required for relaxation of this muscle. In view of the relatively sparse amount of sarcoplasmic reticulum in smooth muscle, an efficient Ca accumulation by mitochondria would be compatible with the needs of the myometrial cell for controlling intracellular Ca. The amounts of sarcoplasmic reticulum and mitochondria in different smooth muscles may vary (Devine, Somlyo and Somlyo, 1972), which suggests that the mechanisms controlling myoplasmic Ca would differ. In addition the importance of extracellular and membrane-bound Ca in the contraction of smooth muscle should not be overlooked.

5. Comparative role of mitochondria and microsomes (FSR) in controlling the myoplasmic calcium in skeletal and smooth muscles

Although it was not one of the original aims of this study to compare Ca binding by the subcellular fractions of smooth and skeletal muscle, the striking differences observed in the affinity of mitochondria and microsomes from the two muscles warrant some attention.

The most important observation in this respect is that mitochondria from skeletal muscle were unable to lower Ca in the external medium below 4×10^{-6} M whereas mitochondria from myometrium were able to reduce Ca down to approximately 10^{-8} M. FSR on the other hand lowered Ca to a very low level (less than 10^{-7} M). Myometrial microsomes were less effective in lowering Ca since the Ca level was reduced to only 3.5×10^{-6} M. This is similar to that observed

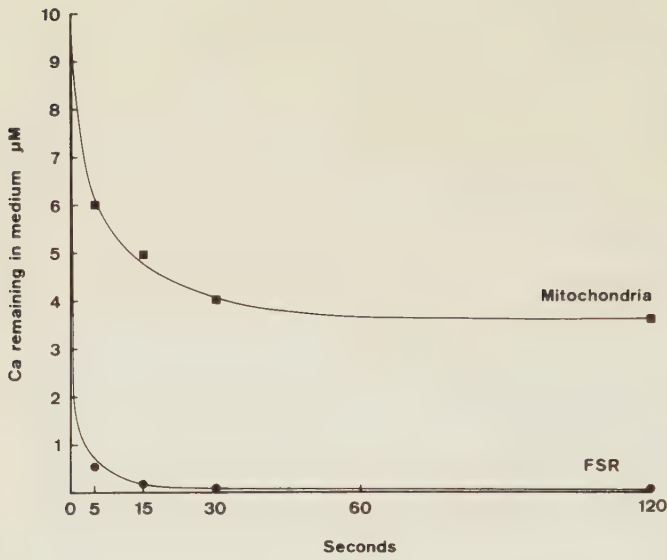
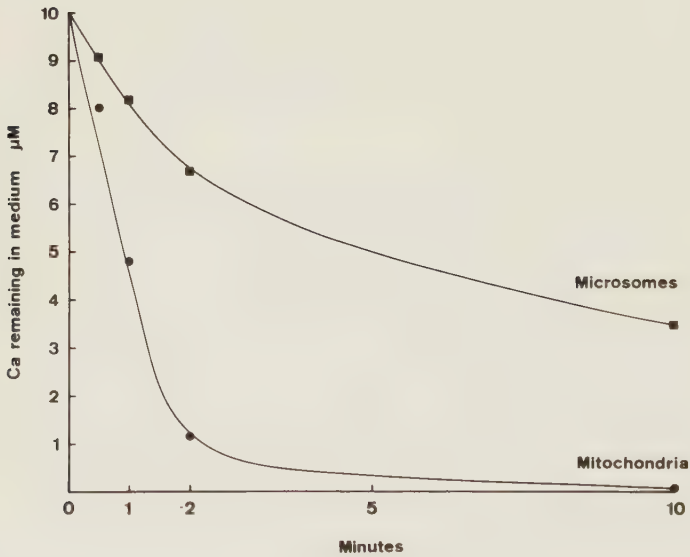


Fig. 1. (a) Ca removal by mitochondria and FSR of frog skeletal muscle from a medium containing 10^{-5} M Ca. Temp. 4°C .



(b) Ca removal by mitochondria and microsomes of human myometrium. Temp. 35°C . (Note also the difference in time scale between a and b).

with mitochondria from skeletal muscle. These differences are illustrated in Fig. 1.

The results of the present study emphasize important differences between skeletal and smooth muscles in fundamental processes such as those regulating myoplasmic Ca. Striking differences in the properties of the contractile proteins of vertebrate skeletal and smooth muscle have been reported and have been recently reviewed by Hamoir (1973).

SUMMARY

Information was sought in the present study on the regulation of Ca level in skeletal and smooth muscle by an investigation of Ca binding by the intracellular structures isolated from the two muscles; the frog leg muscle and the human myometrium.

Various agents which are known to modify the contractile activity of these muscles were tested for their effect on Ca uptake and Ca release by the subcellular particles isolated from the respective muscle. This was done for two reasons: (a) to elucidate the mechanism controlling myoplasmic Ca concentration and (b) to determine the mechanism whereby these agents influence the contractile responses of the intact muscle. The more important findings can be summarized as follows:

Skeletal muscle

1. In contrast to mitochondria, fragmented sarcoplasmic reticulum (FSR) lowered Ca in the medium to very low levels (10^{-7} M).
2. Very low concentrations ($5 \mu\text{M}$) of lanthanum greatly reduced the rate and capacity of Ca uptake by mitochondria. These same concentrations of lanthanum inhibited only the rate of Ca uptake by FSR when the medium Ca was low ($10 \mu\text{M}$) but diminished both the rate and the capacity when the medium Ca was high ($100 \mu\text{M}$).
3. Low concentrations ($5-10 \mu\text{M}$) of Zn failed to affect Ca uptake by either mitochondria or FSR, whereas higher concentration ($25-50 \mu\text{M}$) inhibited Ca uptake by either fraction.
4. Quinidine (1 or 2 mM) released a considerable amount of Ca from mitochondria but had little effect on Ca release from FSR. The uptake of Ca by both mitochondria and FSR was inhibited by higher concentration (1 or

2 mM) of quinidine while the inhibition of mitochondrial Ca uptake was much greater. There was no significant effect on Ca uptake by FSR with lower concentrations (0.4 mM) although a 48 % inhibition of mitochondrial Ca uptake was observed.

5. Chlorpromazine inhibited Ca uptake both by mitochondria and FSR. However, the inhibition of mitochondrial Ca uptake was much greater than that of FSR. High concentrations of chlorpromazine (0.1 mM) released Ca from FSR and mitochondria.
6. Caffeine caused a greater inhibition of Ca uptake by mitochondria than by FSR. However, in contrast to quinidine, caffeine released Ca from FSR and not from mitochondria.

Myometrium

1. Ca uptake capacity of mitochondria was considerably greater than that of microsomes. Oxalate failed to increase Ca uptake by either fraction.
2. Ca uptake by mitochondria could be energized not only by ATP but also by succinate. However, the presence of ATP in the medium was essential for succinate supported Ca uptake.
3. In contrast to microsomes, mitochondria were able to lower Ca in the medium to very low levels (10^{-8} M). In addition the rate of Ca elimination from the external medium was compatible with the speed of relaxation in the intact myometrium.
4. Small concentrations of estrogens inhibited Ca uptake by mitochondria to a greater degree than that observed with cardiac mitochondria. Estrogen was also found to release Ca stored by myometrial mitochondria. An equal concentration of progesterone had no influence on Ca uptake by mitochondria.

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